Protein-Lipid Interactions IV

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To be or not to be in Membrane Domains: Transbilayer Asymmetry and Sphingomyelin-Dependent Preferential Partitioning of the Acetylcholine Receptor

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The preferential partitioning of the nicotinic acetylcholine receptor (AChR) in liquid-ordered (Lo) domains, heterogeneous membrane domains commonly known as rafts, is thought to be a part of its clustering mechanism. Previous studies from our group have shown that AChR lacks preference for Lo domains when reconstituted in sphingomyelin (SM), cholesterol (Chol) and POPC (1:1:1) model systems (Bermúdez et al., 2010). Here we study the effect on the possible Lo-preferential partitioning of purified AChR reconstituted in two different model systems (POPC:Chol, 1:1 and POPC:Chol:SM, 1:1:1) under: a) induced transbilayer asymmetry, resulting from addition of brain sphingomyelin (bSM) to the external hemilayer; and b) the presence of different pure SM species in the model membrane (bSM, 16:0-SM, 18:0-SM or 24:1-SM). AChR distribution was evaluated by fluorescence resonance energy transfer efficiency between the AChR intrinsic fluorescence and Laurdan or dehydroergosterol fluorescence, and also by determining the presence of AChR in detergent-resistant and detergent-soluble domains (1% Triton X-100, 4°C). Both studies show that the induction of transbilayer asymmetry or the presence of 16:0-SM or 18:0-SM, as opposed to bSM or 24:1-SM, leads to a preferential partitioning of AChR in Lo domains. Thus, the localization of AChR in Lo domains strongly depends on the characteristics of the host lipid membrane.

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Formation of Giant Unilamellar Vesicles Containing Active Proteins

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Giant unilamellar vesicles (GUVs), composed of a phospholipid bilayer, are often used as a model for cell membranes. However the study of proteo-membrane interactions in this system is limited because the incorporation of integral and lipid-anchored proteins into the GUVs remains challenging. All pre-existing protocols either produce membranes with a very low protein density or are tailored to specifically support the inclusion of a particular protein. We recently developed a simple generic method to incorporate protein in GUVs. It does not require specific lipids or reagents, works in physiological conditions with high concentrations of protein, and the resulting proteo-GUVs can be micromanipulated. Moreover, our protocol is not limited to a narrow range of protein substrates; indeed we have already successfully incorporated two trans-membrane proteins and one lipid-anchored peripheral protein.
These first proof-of-principle proteins present different types of challenges and thus demonstrate the broad utility of our method. TolC is an integral membrane protein and part of a heterotrimer, that together comprise a major multidrug efflux pump in E coli. The neuronal t-SNARE is a protein complex with a single transmembrane domain that mediates membrane fusion. Because of its propensity to aggregate t-SNARE is usually not functional after insertion in GUVs. To study lipidated proteins, we incorporated a modified form of the autophagy protein GABARAP L1, which we anchored it to the membrane via a cysteine-maleimide covalent bond. In each case, we verified that the proteins remain active after incorporation. We also verified their mobility by performing diffusion measurements via fluorescence recovery after photo bleaching (FRAP) experiments on micromanipulated GUVs. The diffusion coefficients are in agreement with previous data.

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Influence of a Central Tryptophan and of Cholesterol on the Properties of Defined Transmembrane Helical Peptides

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GWALP23 (acetyl-GGALW^{5}LALALALALALW^{19}LAGA-amide) provides a favorable host framework for investigations of the influence of guest amino acids, for example a third, centrally located, Trp residue, within the hydrophobic core of a well characterized transmembrane helix. It is crucial to note that the orientation and rotation of GWALP23 are sensitive to single-residue replacements, in part because the membrane-spanning helix exhibits only limited dynamic averaging of solid-state NMR observables such as the ^{2}H quadrupolar splitting (Biophys. J. **101**, 2939). We introduced a Trp residue into position 12 or 13 of GWALP23 (replacing either L12 or A13) and incorporated specific ^{2}H-Ala labels within the helical core sequence. Solid-state ^{2}H NMR spectra of GWALP23-W12 reveal that the peptide remains helical and retains a dominant preferred tilted transmembrane orientation with only a low extent of dynamic averaging, comparable to GWALP23 itself. Indeed, the tilt and dynamics of GWALP23-W12 are quite similar to the values observed for GWALP23 in DMPC bilayer membranes. We have analyzed the dynamics of the peptide helices using a modified Gaussian treatment as well as a semi-static treatment. The results indicate that a central Trp residue at position 12 does not appreciably perturb the properties of bilayer-spanning GWALP23. (By contrast, Arg-12 or Lys-12, when charged, induces multi-state behavior for GWALP23 in bilayer membranes [PNAS **110**, 1692].) Additionally, we are investigating the influence of cholesterol upon the properties of membrane-spanning GWALP23, GWALP23-W12 and GWALP23-K12.

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Regulation of K-RAS Membrane Association: Calmodulin Versus PDEδ

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K-Ras is a small GTPase that plays a critical role in human cancer cell biology. Selective membrane localization and clustering of K-Ras4B into microdomains are mediated by its polybasic farnesylated C-terminus. The importance of the subcellular distribution for the signaling activity of K-Ras4B became apparent from recent *in vivo* studies [1]. PDEδ and the Ca^{2+}-binding protein calmodulin (CaM) are known to function as potential binding partners for farnesylated Ras proteins, leading to a modulation of the spatiotemporal organization of K-Ras4B. The latest study of our group showed that PDEδ is not able to extract K-Ras4B from model raft membranes; instead, an effective delivery of PDEδ-solubilized K-Ras4B to the plasma membrane was proposed [2]. Compared to PDEδ, CaM exhibits additional interaction sites to the G-domain of K-Ras4B and was shown not to be required for the transport of K-Ras4B to the plasma membrane. Thus, it was suggested that calmodulin dissociates K-Ras4B from membranes [3]. However, the exact role of CaM in the intracellular localization and dynamics of K-Ras4B still remains elusive.
In the present approach, the influence of CaM on the interaction of GDP- and GTP-loaded K-Ras4B with anionic model raft membranes has been investigated by a combination of different spectroscopic and imaging techniques. The results suggest that binding of the acidic CaM to the polybasic stretch of K-Ras4B reverses its charge, leading to repulsion of the complex from anionic membranes. Since one farnesyl anchor alone is not sufficient to stably anchor Ras proteins to membranes, CaM would be able to dissociate K-Ras4B from plasma membranes, contrary to PDEδ.

References:

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Influence of Glutamic Acid Residues on the Properties of Model Membrane-Spanning Helices

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GWALP23 (acetyl-GGALW^{5}LALALALALALW^{19}LAGA- amide) is a constructive model peptide for investigations of single-residue effects on protein-lipid interactions and the properties of membrane-spanning helices (J.Biol. Chem. 285, 31723). GWALP23 has favorable properties in bilayer membranes because the peptide exhibits only limited dynamic averaging of NMR observables such as the ^{2}H quadrupolar splitting or the ^{15}N-^{1}H dipolar coupling (Biophys. J. 101, 2939). To investigate the potential influence of negatively charged glutamic acid side chains upon system properties, we have substituted a single Leu residue with Glu at different positions and incorporated specific ^{2}H-Ala labels in the core of the single-Trp peptide Y^{5}GWALP23 (see Biochemistry 51, 2044). Solid state ^{2}H NMR experiments showed well defined ^{2}H quadrupolar splittings for Y^{5}GWALP23-E16 in the pH range from 4.0 to 9.0, suggesting that the peptide helix is well oriented in DOPC lipid bilayers. The E16-containing peptide seems to exhibit multi-state behavior at pH 10.9, in bilayers formed by ether-linked lipids, suggesting a pKa that is above pH 9 for the E16 side chain. The rather modest shift in the ^{2}H quadrupolar splittings suggests that the orientation of the transmembrane peptide helix changes rather little at high pH. It is conceivable that the close proximity of E16 to W19 could provide stability to the neutral peptide helix and perhaps influence the pKa of E16. The molecular cousin having E14 instead of E16 shows multi-state behavior from pH 4.0 to pH 10.9 rendering pKa

determination enigmatic at this time. Some puzzles remain with respect to comparisons between E16 and E14. We are additionally investigating the peptide-lipid behavior when Glu is introduced in position 12, at the peptide center.

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Influence of pH and Histidine Residues on Membrane-Spanning Helical Peptides

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Synthetic model peptides such as GWALP23 (acetyl-GGALW^{5}LALALALA LALALW^{19}LAGA-amide)provide a useful host framework for investigations of the influence of polar amino acids, for example histidine residues, within the hydrophobic core of a transmembrane helix. Importantly, membrane-spanning GWALP23 is quite sensitive to single-residue replacements, in part because the transmembrane helix exhibits only limited dynamic averaging of solid-state NMR observables such as the ^{2}H quadrupolar splitting (Biophys. J. 101, 2939). We inserted His residues into position 12 and/or 13 of GWALP23 (replacing either L12 or A13) and incorporated specific ^{2}H-Ala labels within the helical core sequence. Solid-state ^{2}H NMR spectra of GWALP23-H12 reveal a marked difference in peptide behavior between acidic and neutral pH conditions. At neutral pH, GWALP23-H12 exhibits a well-defined tilted transmembrane orientation in both DOPC and DLPC bilayer membranes. To prevent the acid catalyzed degradation of lipids, we employed ether-linked DOPC bilayers to observe the effect of low pH on the L12H mutant. Under acidic conditions GWALP23-H12 is highly dynamic and exhibits multiple states. Indeed, the multi-state behavior of GWALP23-H12, when His is charged between pH 1.5 and pH 3, resembles closely that of GWALP23-R12 at neutral pH (J. Am. Chem. Soc. 132, 5803). The dramatic change in the behavior of GWALP23-H12 indicates a pK_a value less than 3 for His near the center of a lipid bilayer. Investigations are in progress to chemically exchange the C2 imidazole hydrogen of His for deuterium in the peptide, toward a goal of enabling direct observation of the His ring by solid-state ^{2}H NMR over a range of pH and buffer conditions.

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Molecular Insight for the Effect of Lipid Raft on Thrombospondin-1 and Calreticulin Interactions

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Thrombospondin-1 (TSP1) binding to calreticulin (CRT) on the cell surface stimulates association of CRT with LDL receptor-related protein (LRP1) to signal focal adhesion disassembly and engagement of cellular activities (J. Biol. Chem. 275:36358-68, 2000; J. Biol. Chem. 277: 37219-28, 2002). Study demonstrated that lipid rafts are necessary for TSP1-mediated focal adhesion disassembly (J. Biol. Chem. 279, 23510-16, 2004), but the molecular mechanism of the phenomenon is still unknown. In this study, we investigated the interactions of a lipid bilayer and a lipid raft with CRT and TSP1-CRT complex and their effects on the structural changes of CRT and TSP1-CRT complex via atomically detailed molecular dynamics simulations. The lipid bilayer was modeled as a 1-palmitoyl-2-oleoyl-sn-glycero-3phosphocholine (POPC) bilayer with 1152 lipids. The lipid raft was modeled as a bilayer of POPC lipids mixed with cholesterol (CHOL) (40% of CHOL molecules in the lipid raft) or with both CHOL and sphingomyelin (SM) (the ratio of the number of POPC lipid, CHOL and SM is: 3:4:3). Results showed that TSP1 binding to CRT resulted in a more ''open'' conformation for CRT P-domain with respect to the CRT N-domain compared to that of single CRT in a lipid raft environment, but not in a POPC bilayer environment. Sphingomyelin enhanced the ''open'' CRT conformation by TSP1, which could expose the potential binding site(s) in CRT for binding to LRP1 to signal focal adhesion disassembly. Results also showed that micro- and mesoscopic properties of a lipid raft were significantly different from a POPC bilayer, which could also affect cell surface CRT interactions with TSP1. Results from this study provided molecular insight for the effect of lipid raft on TSP-CRT interactions and CRT-mediated focal adhesion disassembly.

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Analysis of the Molecular Organization of Lipoprotein-Associated Apolipoprotein E, an Anti-Atherogenic Protein

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Human apolipoprotein E3 (apoE3) is a 299 residue exchangeable apolipoprotein that has the ability to exist in lipid-free and lipoprotein-bound states. It is composed of an N-terminal domain bearing LDL-receptor binding sites and a C-terminal (CT) domain bearing high-affinity lipid-binding sites; the latter is also responsible for mediating cellular cholesterol efflux. ApoE plays a crucial role as an anti-atherogenic agent in atherosclerosis by virtue of its ability to mediate reverse cholesterol transport by promoting cholesterol efflux from macrophages, a process that leads to the initial formation of nascent discoidal high-density lipoproteins (nHDL). The objective of this study is to understand the molecular organization and conformation of apoE3 (1-299) and isolated CT domain (201-299) on reconstituted HDL (rHDL) and nHDL generated by macrophages. To accomplish this, we over-expressed and purified recombinant apoE3 and the CT domain bearing single Cys at selected sites. rHDL was prepared using unlabeled or pyrene-labeled single Cys variants and phospholipids, while nHDL was generated by exposing cholesterol-loaded J774 macrophages to unlabeled or labeled apoE bearing probes at defined sites. Lipid-associated apoE was obtained by density gradient ultracentrifugation. The relative organization of apoE molecules on rHDL and nHDL was followed by monitoring pyrene excimer fluorescence and by site-specific cross-linking studies, both complementary validators of spatial proximity at ~10 Å distance. The appearance of excimer emission and cross-linked dimers regardless of the location of the single Cys is indicative of pairs of apoE3 molecules oriented parallel to each other on the HDL to form a dimer. Based on our results, we propose that apoE molecules circumscribe a bilayer of lipids adopting a belt-like conformation. Our study offers mechanistic details of apoE interaction with lipids, which aids in understanding its role in cardiovascular disease.

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Dual-Color Fluorescence Cross-Correlation Spectroscopy of Reconstituted Protein-Membrane Systems

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Liposomes are commonly used as model membranes for studying the function of isolated membrane proteins. They are also used for reconstituting large membrane-associated protein complexes, such as the protein coats of intracellular transport vesicles. Here, we show examples of how dual-color fluorescence cross-correlation spectroscopy (or, more generally, fluorescence fluctuation spectroscopy) can serve to optimize in vitro reconstitutions and unravel protein-membrane interactions:

(A) The reconstitution of detergent-solubilized, purified membrane proteins into liposomes is a prerequisite for a myriad of experimental studies, but experimental conditions generally have to be determined on a case-by-case basis. Fluorescence correlation spectroscopy (FCS) distinguishes micelles, liposomes and aggregates in homogeneous and heterogeneous samples based on their different mobilities. Dual-color fluorescence cross-correlation spectroscopy (dcFCCS), an extension of FCS, additionally detects the co-localization of protein and lipid in these diffusing entities, facilitating the optimization process [1]. The principle is not limited to proteoliposome formation, but can be applied more generally to reconstitutions into small diffusing membrane entities, such as liposomes or nanodiscs.

(B) Studies of large, multi-subunit protein complexes, such as the COPII transport vesicle coat, require analysis of protein-lipid as well as protein-protein interactions. By virtue of their low perturbation, fluorescence techniques help optimizing complex reconstitution systems to obtain suitable preparations for cryo electron microscopy analysis of a complex structure [2], and help expanding the complexity of the reconstitution. The interaction of COPII components among each other and with model membranes are investigated.

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Divalent Cation- and Cholesterol-Induced Perturbation in Lipid Lateral Organizations and Polyphosphoinositide-Protein Interactions

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Phosphatidylinositol 4,5-bisphosphate (PIP2) controls many important cellular events. A major challenge in understanding how PIP2 function in vivo is to define its physical state and lateral organization in cell membranes. The hypothesis that PIP2 forms nano-sized clusters in the presence of intracellular divalent cations by electrostatic interactions was examined in model membranes with or without cholesterol-mediated phase segregation. Additional studies show how